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REMARKS

The Office Action and the cited and applied reference have been carefully reviewed. No claim is allowed. Claims 11-16, 18-21 and 32 presently appear in this application, with claim 32 being newly added in place of canceled claim 17, and define patentable subject matter warranting their allowance. Reconsideration and allowance are hereby respectfully solicited.

The personal interview among the undersigned and Examiners Bui and Helmer on October 9, 2003, is hereby gratefully acknowledged. The undersigned wishes to thank the examiners for the courtesies extended during this interview. The proposed amendments discussed at the interview are presented herein.

Claim 18 has been rejected as being directed to non-statutory subject matter. This rejection is obviated by the amendment to claim 18 to recite "wherein said isolated tissue comprises the cytokine expressed in said transgenic plant.

Claims 11-21 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. This rejection is believed to be obviated by the amendments to the claims.

In amended claim 11, support for the new recitations are found on page 20, first full paragraph, and the Examples (grown-up), page 4, lines 6-14 (cytokine), page 5, line 11 to

page 6, line 3 (regulatory sequences), pages 9-10 (calli), and page 12, first full paragraph (0.1 ng to one milligram per one kilogram by fresh weight of the grown up plant). In amended claims 14, 15 and 31, the recitations of "the transgenic plant is ingested by a mammal belonging to the same family of the mammalian source used" and "can be ingested by… without cooking or heating" are supported by the specification at page 4, first full paragraph, and at page 13, respectively.

Reconsideration and withdrawal of this rejection are therefore respectfully requested.

Claim 13 has been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such as way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is obviated by the deletion of the recitation held by the examiner to be unsupported in the specification. However, it should be pointed out that the Goodman et al. U.S. Patent 4,956,282, relied upon by the examiner below as prior art listed examples of well known "growth factors" at column 3, lines 24-27. Accordingly, the term "growth factors" is well recognized and understood in the art.

Claims 11-21 remain rejected under 35 U.S.C. §102(b) as being anticipated by Goodman et al. (U.S. Patent No. 4,956,282 for reasons of record. This rejection is respectfully traversed.

Claim 21 is amended to make clear that the food composition of claim 20 is "further supplemented with trehalose". Accordingly, trehalose is not inherently present in the plant but must be added as a supplement to the food composition.

Regarding the disclosure in Goodman at column 5, lines 40-60:

> When the plants have been grown to the desired stage, the plants or plant parts, e. g., seeds, fruit or the like, may be harvested .... The plants may be ground and extracted .... Where the product can have a physiological effect on ingestion, it may be sufficient that the product be retained with the plant. This will be true where the plant part is edible, such as fodder which could include bovine growth hormone, seed, nuts, fruit and vegetables. (emphasis added),

applicants would like to point out the fact that all the statements in the cited paragraph are expressed with the words "may be". This means that the statements in the paragraph were made based on Goodman's hope or speculation, and did not rely on experimental results. In fact, the experimental results as shown in the "EXPERIMENTAL" section at columns 5 to 10 of Goodman does

not refer to "grown-up" transgenic plants. The results stop at the stage where callus is obtained.

As argued previously, Goodman's invention was made to overcome the disadvantages of the conventional recombinant interferon producing technique in which microorganisms, such as *Escherichia coli,* are used, and provides plant cells in place of microorganisms, to recombinantly produce interferon. To attain this, grown-up transgenic plants are not necessary. Callus, as an interferon producing factory, is enough to produce interferon. This situation is easily understood from the nature of Goodman's invention.

Furthermore, it should be noted that actually obtaining a grown-up plant from callus requires undue experimentation because there are various factors and conditions which have to be settled in order to grow callus into a desired plant. To show this, attached hereto are three documents;

1. "Plant Cell Culture", edited by R A Dixon, IRL Press, (1985) pp. *79-105*. This document states that "explant" selection plays a paramount part in successful plant regeneration studies paragraph *1.2* beginning from the bottom of page *80)* and further states that the composition of the nutrient medium is the next important parameter which must be optimized in order to

obtain successful plant regeneration (paragraph *1.3* beginning from page *83)*. In particular, as shown in Table *4* at pages *85-90*, the medium requirements for callus, shooting and rooting are different depending on plant species.

2. "Journal of Plant Nutrition", *(1994)*, vol. 17, no. *4, pp. 549-560*. This document shows that salt tolerance screened at the callus level is not expressed in regenerated plant. This implies the difficulty of expecting the characteristics of regenerated plants from those of callus level.

3. "Heredity and Breeding of Plants", edited by Hiroshi Ikehashi, published by Yoken-do Publisher (2000), pp. 200-201, along with an English translation of the relevant portion. This document states the same fact as shown in the document 2.

In view of the above, it is apparent that regenerating a grown-up plant from callus requires undue experimentation. In this regard, it is submitted that Goodman fails to disclose a grown-up transgenic plant in such a way as to reasonably convey to one skilled in the art that Goodman had possession of a grown-up transgenic plant.

In addition, claim 11 is now amended to make clear that a DNA sequence, being introducing into a plant protoplast, etc., has an additional constitutive regulatory sequence, inducible

regulatory sequence, or tissue-specific regulatory sequence. In Example 1 at pages 15-19, a DNA having "cauliflower mosaic virus 35S promoter" (see page 16, line 11) as "constitutive regulatory sequence" is used to regenerate transgenic tomato.

By contrast, Goodman never teaches using such DNA at all. Claims 11-20 are not anticipated by Goodman et al.

Example 1 shows that a grown-up transgenic plant, i.e., tomato, was obtained and confirms that interferon-α was expressed in the grown-up tomato. Example 1 further investigated pharmaceutical functions of the transgenic tomato fed to mice and obtained experimental animal results showing that the transgenic tomato actually enhanced the immunological potency of mice that ingested the transgenic tomato. Transgenic carrot, lettuce and strawberry were obtained in Examples 2 to 4 and similar results were obtained.

By contrast, Goodman does not disclose any grown-up transgenic plant that were actually regenerated, or any experimental results as to a grown-up transgenic plant. It would have been difficult even for one of skill in the art to reasonably understand that Goodman actually discloses a grown-up transgenic plant including its industrial usefulness.

Reconsideration and withdrawal of the rejection are therefore respectfully requested.

In view of the above, the claims comply with 35 U.S.C. §112 and define patentable subject matter warranting their allowance. Favorable consideration and early allowance are earnestly urged.

Respectfully submitted,

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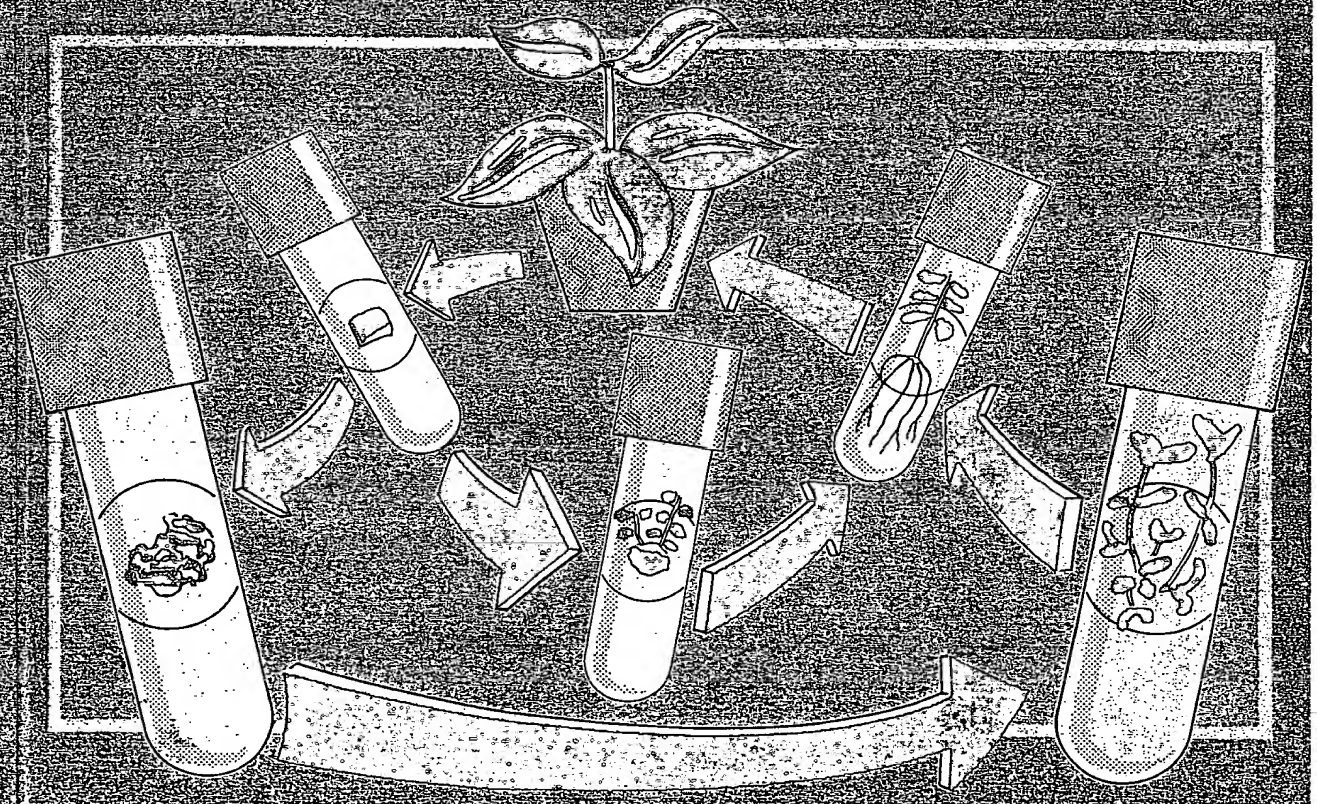
N-1

Plant cell culture

a practical approach

Edited by
R A Dixon



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CHAPTER 5

Embryogenesis, Organogenesis and Plant Regeneration

B. TISSERAT

1. INTRODUCTION

The procedures of plant tissue culture have developed to such a level that any plant species can be regenerated *in vitro* through several methodologies. The rate of plant regeneration in tissue culture varies greatly from one species to another (1–5). Various cells, tissues and organs from numerous plant species can be cultured successfully to regenerate whole plants (1–15). There may be several processes of plant regeneration for each species but usually only one type is most efficient (4,5). Methods to regenerate plantlets have developed from a myriad of independent studies reported in numerous publications (6,8,10,15–23). The purpose of this chapter is to present a workable plant tissue culture propagation guide. A comprehensive review is not necessary because: (a) prior reports are numerous and sometimes conflicting; and (b) knowledge and techniques of plant regeneration, as with every other field of tissue culture, are changing and improving thus often relegating prior work to insignificance.

Plant regeneration is the cornerstone of tissue culture methodology. Without plant regeneration protoplast hybridisation, growth and differentiation studies, generation of genetic variable plants, anther culture, commercial cloning for the purpose of rapid multiplication of desirable or difficult to propagate species, disease-elimination through meristem culture, and many growth regulator studies would become impotent. Through the knowledge of plant regeneration these disciplines have now become powerful tools for agricultural and horticultural research.

In this chapter, several techniques will be identified and presented to represent the current methodology for propagation of plants in sterile culture. It should be noted that these are adequate methods but do not necessarily represent the optimum or the most efficient procedures. Plant propagation procedures change as knowledge of this subject increases and improves. Presentation of a series of case histories is not necessary to acquaint potential investigators with the fundamentals of plant regeneration in tissue culture. The procedures presented will yield quick and reliable results in relation to propagation of most species *in vitro*. Some alterations in techniques and media compositions may be necessary for some species (*Table 4*). Several reviews dealing with plant regeneration have been written in the last few years (1–6,8–13,15,17–19,23–25). These reviews could

further serve as source material to interested investigators seeking a broader knowledge of the subject or to those requiring introductory information for particular species as a basis for conducting more extensive studies.

1.1 Modes of Plant Regeneration

The term plant tissue culture has generated into an all encompassing, convenient term to describe all types of sterile plant culture procedures pertaining to the growth of plant protoplasts, cells, tissues, organs, embryos and plantlets. Plant regeneration through tissue culture will be used here to describe the production of plantlets in sterile culture. The term *in vitro* is used to describe the sterile, artificial culture environment; correspondingly, the term *in vivo* relates to the natural non-sterile conditions. The term plantlet is used to describe the sterile plant with a distinct root and shoot system developed *in vitro*.

Plant regeneration through tissue culture can be accomplished using one of three methods: embryo culture, somatic embryogenesis and organogenesis. Embryo culture is the aseptic culture of a zygotic embryo. The embryo is excised from either the seed or ovule and planted on a substitute endosperm environment (i.e., nutrient medium). Subsequent embryo development and germination occurs as it would from the seed. This type of plant regeneration is not discussed in this chapter although many of the principles and procedures employed in its use overlap with the other methods of plant regeneration. Somatic or asexual embryogenesis is the production of embryo-like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. Such embryos can further develop and germinate into plantlets through the events that correspond with the zygotic occurrences (15). This phenomenon can occur in several plant species *in vitro* from the culture of various cell types, tissues and organs while in nature such events are usually intra-ovular occurrences (15). Plant development through organogenesis is the formation and outgrowth of shoots from callus or initiation and outgrowth of axillary buds generated from cultured tips, and their subsequent adventitious rooting. The shoot is a unipolar structure and is physically connected to the tissue of origin. Occasionally, roots may give rise to shoots. Several types of plant regeneration methods may be available for the propagation of some plant species (*Figure 1*).

Duplication of experimental procedures reported in the literature is not always possible. Reported research techniques may be preliminary or fragmentary in nature. Lack of standardisation of techniques among researchers greatly contributes to conflictive and non-reproducible results. The following factors will contribute substantially to successful plant propagation studies: facilities, medium composition, culture environment, technique skill and explant selection.

1.2 Explant Factors

The term explant is used to describe the initial piece of the plant introduced *in vitro*. Explant selection plays a paramount part in successful plant regeneration studies. For optimum success, explants must be obtained from healthy vigorous

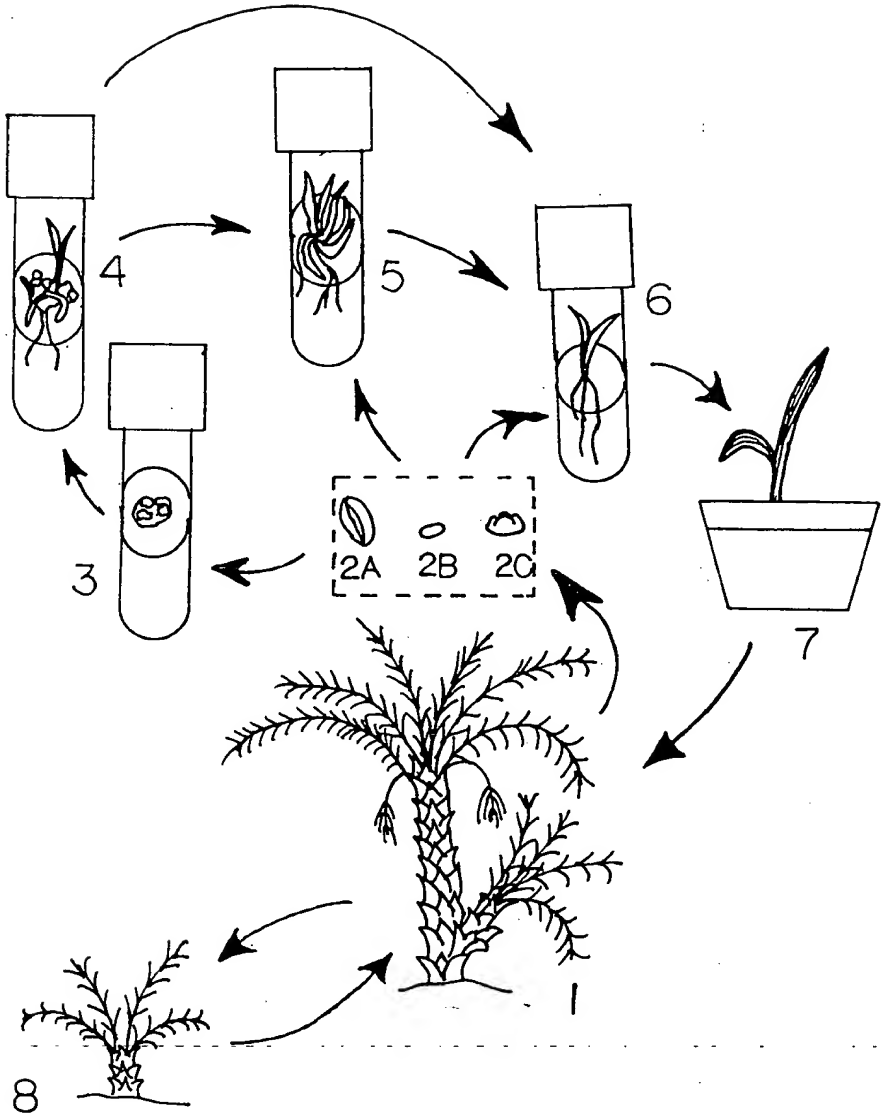

Figure 1. Morphogenetic potential of the date palm *in vitro*. (**1**) In nature, date palms are propagated through seed germination or rooting offshoots (**8**). (**2**) Various explants such as lateral buds (**2A**), excised embryos (**2B**) and shoot tips (**2C**) can be cultured and used to produce plantlets. A number of regeneration methods such as: embryo culture (**6**), organogenesis *via* axillary budding (**5**), or somatic embryogenesis (**3** and **4**) can be employed. Note that these events can intertwine. (**3**) Explants planted on medium containing 0.45 μM 2,4-D produce nodular callus and somatic embryo formation after 2–6 months in culture. (**4**) Transferring callus to medium devoid of 2,4-D results in macro-embryo formation and germination. (**5**) Plantlets and shoot derived from zygotic and somatic embryos, lateral buds, and shoot tips cultured on medium containing 0.54 μM NAA and 44.4 μM BA produce axillary bud outgrowths. (**6**) Rooting of lateral buds, shoot tips and plantlets on medium containing 0.54 μM NAA. (**7**) Free-living palms obtained through tissue culture.

plants. Pre-culture plant conditions may greatly influence the subsequent growth of explants in culture (e.g., rate and nature of chemical fertiliser treatment, season and environmental growth conditions). Practically any part of the plant can be successfully cultured *in vitro* and can regenerate plantlets provided the explant is obtained at the proper physiological stage of development. Immature tissues and organs are invariably more morphogenetically plastic *in vitro* than mature tissues and organs. Furthermore, meristematic tissues or organs should be selected in

Table 1. Sources of Angiosperm Explants and their Relative Morphogenic Potential *In Vitro.*

Explant type	*Morphogenic Potential/Availability* [a]			
	Dicotyledons		*Monocotyledons*	
	Herbaceous	*Perennial*	*Herbaceous*	*Perennial*
Shoot tip	high/abund.	high/abund.	high/abund.	high/one
Lateral buds	high/abund.	variable/abund.	high/abund.	variable/few
Inflorescence	high/abund.	variable/abund.	high/abund.	variable/abund.
Rachis	high/abund.	low/abund.	variable/abund.	low/abund.
Mature leaf	high/abund.	low/abund.	variable/abund.	low/abund.
Immature leaf	high/abund.	variable/abund.	variable/abund.	variable/abund.
Petiole	high/abund.	low/abund.	high/abund.	low/abund.
Root	high/abund.	low/abund.	high/abund.	low/abund.

[a] Morphogenic potential is used to describe the ability of the explant to regenerate plants *in vitro*. Availability refers to the relative number of explants obtainable from a single plant. The term abund. is an abbreviation for abundant.

preference to other tissue sources because of their clonal properties, culture survival, growth rates and totipotentiality *in vitro* (*Table 1*). Meristems, shoot tips, axillary buds and immature, rapidly growing tissues are particularly suited as choice explants. Mature leaves, roots, stems, petioles and flower parts from herbaceous species can often be successfully cultured to initiate plantlets through either the organogenesis or somatic embryogenesis processes (1–3,6,10,17–19,23). Herbaceous explant plasticity contrasts markedly with the growth of the same type of explants obtained from perennial species and some cereals.

Following explant selection, the disinfection procedure becomes the primary consideration (*Table 2*). Disinfection of tissues is necessary in order to eradicate surface microorganisms. The presence of any contaminant will interfere with the growth of explants or cultures. Fungal and bacterial explant contamination in plant cultures is usually detectable 1–14 days after planting. Contaminated cultures should be discarded immediately since their presence only leads to further air-borne contamination dissemination in the culture room. Spore dormant microorganisms occurring on the explant surface are resistant to most disinfectants. The incorporation of antibiotics into the medium or the dipping of explants in antibiotics has been advocated by some researchers. This practice is usually unreliable and tedious and is not recommended. The occurrence of contamination means failure of the surface sterilisation method effectively to eliminate microorganisms. Microorganism survival following surface sterilisation could be due to the type of explant employed, disinfectant type and/or treatment time employed. Prior washing of the explants with soap and water or dipping in ethanol is recommended to induce adequate wetting and initial cleaning. Most commonly, a dilute solution of sodium hypochlorite (0.25–2.63%) is used as a disinfectant. An emulsifier such as Tween-20 (polyoxyethylene sorbitan monolaurate) is added at the rate of 1 drop per 100 ml of solution. Vegetative explants are usually sterilised for 10–20 min and may be rinsed with sterile water to remove residual disinfectant. Mechanical agitation using a stirrer and/or application of vacuum is sometimes helpful to dislodge air bubbles and facilitate an even distribution of the

Table 2. Disinfectant Methods for the Surface Sterilisation of Explants for Plant Regeneration Studies.

Explant type	*Disinfectant* [a] *(concentration)*	*Procedure*
Leaves, flowers, stem	NaOCl (0.26–2.6%)	Wash tissue with mild detergent in water; decant; incubate 10–20 min in 1.3% NaOCl; decant; rinse three times with sterile water. This procedure is sufficient to surface sterilise most explants.
Leaves	NaOCl (0.25%)	e.g., tobacco. Wash with mild detergent in water; rinse with 70% ethanol; decant; incubate 20 min; decant; rinse three times with sterile water.
Roots	NaOCl (2.6%)	e.g., date palm. Incubate 30 min; no sterile water rinse.
Tap root	$HgCl_2$ (0.1%)	e.g., carrot. Cut root in 50 mm long pieces, incubate 30 min; decant; rinse three times in sterile distilled water.
Seeds	NaOCl (0.25–2.6%)	e.g., tomato. Incubate seeds 1 min in 80% ethanol; decant; soak for 10 min in 1.6% NaOCl; decant; rinse three times in sterile distilled water.
		e.g., tobacco. Incubate seeds 10 min in 0.25% NaOCl; decant; rinse three times with sterile distilled water.
	Iodine (3%), $HgCl_2$ (0.5%), HgI_2 (0.5%), or $HgBr_2$ (0.5%)	e.g., date palm. Incubate seeds 30 min; decant; no sterile water rinse.

[a]Prepare all solutions with water except iodine which is dissolved in 95% ethanol. Add emulsifier to all NaOCl solutions (1 drop per 100 ml of solution). Disinfectant solutions should be agitated periodically by hand or rotated continuously on an orbital shaker at 25–50 r.p.m. throughout incubation.

disinfectant over the explant. When one type of disinfectant procedure fails to achieve adequate explant sterility others should be attempted.

An increasing body of evidence suggests that internally lodged contaminants sometimes may occur within the explants and subsequent established cultures. In some cases, bacterial cultures (e.g., *Pseudomonas* in broccoli) may co-exist with plant cultures and remain visually undetected for several culture passages while adversely reducing the multiplication rates and other growth responses (7). Some microorganisms (e.g., bacteria in the spore stage) may remain latent for several recultures before proliferating. The relationship, if any, between these latent contaminants and normal development is not understood.

1.3 **Nutrient Medium Factors**

The composition of the nutrient medium is the next important parameter which must be optimised in order to obtain successful plant regeneration. A multitude of plant tissue culture media are suitable for plant regeneration (18). The formulation developed by Murashige and Skoog (MS) in 1962 is the most common medium employed in the plant tissue culture field (*Table 3*). *Table 4* gives selected examples of the wide number of species that can be regenerated using this basic medium with varying growth regulator levels. Adverse explant chemical discharges, often referred to as browning, can usually be combatted through frequent reculturing, or inclusion of an adsorbent such as activated charcoal or polyvinylpyrrolidone in

Table 3. Composition of Murashige and Skoog Plant Tissue Culture Medium and Alternative Vitamin Sources.

Component	*Concentration* [a] *(μM)*
Inorganic salts	
Macronutrients:	
NH_4NO_3	20 600
KNO_3	18 800
$CaCl_2.2H_2O$	3000
$MgSO_4.7H_2O$	1500
KH_2PO_4	1250
Micronutrients	
KI	5.0
H_3BO_3	100
$MnSO_4.4H_2O$	100
$ZnSO_4.7H_2O$	30
$Na_2MoO_4.2H_2O$	1.0
$CuSO_4.5H_2O$	0.1
$CoSO_4.6H_2O$	0.1
Na_2EDTA	100
$FeSO_4.7H_2O$	100
Carbohydrate source	
Sucrose	88 000
Vitamin sources	
Meso-Inositol dihydrate	0.46
Thiamine·HCl	1.2
or Modified White's vitamins	
Thiamine·HCl	0.59
Pyridoxine·HCl	4.9
Nicotinic acid	8.1
Glycine	53.28
or Nitsch and Nitsch vitamins	
Thiamine·HCl	1.48
Pyridoxine·HCl	0.24
Nicotinic acid	40.61
Glycine	26.64
Folic acid	1.13
Biotin	0.2
Complex addenda	
Agar (%)	0.8

[a] For concentrations of stock solutions, etc., see Chapter 1, *Table 1.*

the medium. When adsorbents are employed the concentrations of other medium ingredients (e.g., growth regulators) are altered significantly

Given the use of a single culture medium (e.g., MS medium) varying the growth regulator levels and types often determines the route of morphogenesis *in vitro*. Generally, medium containing high auxin levels will induce callus formation. Inclusion of cytokinins with auxins may be beneficial for promotion of callus formation in some species. Lowering the auxin concentration and increasing the cytokinin concentration is traditionally performed to induce shoot organogenesis

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Araceae					
Anthurium andraeanum (2)	Leaf	O-C	0.4 2,4-D, 3.2 PBA	3.2 PBA	0.054 NAA or –
Aracaceae					
Phoenix dactylifera (6)	Axillary bud	SE-C	135 2,4-D, 13.9 2iP 0.03% charcoal	–	0.54 NAA
Aquifoliaceae					
Ilex aquifolium (6)	Embryo	SE-D	–	–	–
Araliaceae					
Hedera helix (2)	Stem	O-C	21.8 NAA, 9.3 K	5.4 NAA, 2.3 K, 200 mg/l CH	0.054 NAA
Begoniaceae					
Begonia x *hiemalis* (21)	Leaf	O-D	–	0.17 BA, 0.54 NAA	0.54 NAA
Brassicaceae					
Brassica campestris (16)	Node	AS	4.4 BA	4.4 BA	0.44 BA, 5.4 NAA
Bromeliaceae					
Ananas sativus (1)	Axillary bud	O-D	–	9.7 NAA, 9.8 IBA, 9.8 K	0.054 NAA or –
sativus (2)	Axillary bud	O-C	28.1 NAA, 29.7 IBA, 9.8 K	9.7 NAA, 9.8 IBA, 9.8 K	0.054 NAA or –
Betulaceae					
Betula alleghaniensis (16)	Shoot tip	AS	22 Z	22 Z	4.9 IBA
Cactaceae					
Mammillaria woodsii (2)	Stem	O-D	–	9.8 IBA, 9.3 K	0.054 NAA
Caricaceae					
Carica stipulata (6)	Peduncle	SE-C	1 NAA, 2 BA	1 NAA, 2 BA, 1% charcoal	0.054 NAA
Caryophyllaceae					
Dianthus caryophyllus (10)	Shoot tip	AS	4.4 BA, 0.057 IAA	4.4 BA, 0.057 IAA	0.57 NAA

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Compositae					
Chrysanthemum morifolium (2)	Shoot tips, petals	O-C	46.5 K, 5.4 NAA	9.3 K, 0.11 NAA, 29 GA	0.054 NAA or –
morifolium (16)	Shoot tip	AS	9.3 K, 0.11 NAA	9.3 K, 0.11 NAA	–
Cichorium endiva (2)	Embryo	O-C	27 2,4-D	0.19 K	0.054 NAA
Stevia rebaudiana (2)	Seed, leaflets	O-C	53.7 2,4-D 9.3 K	4.4–8.8 IAA	–
rebaudiana (2)	Nodal segment	AS	8.9 BA	8.9 BA	54 NAA
Crassulaceae					
Crassula argentea (2)	Leaf	O-C	100 2iP	0.1 BA	0.054 NAA
Cruciferae					
Arabidopsis thaliana (2)	Leaf, stem, seed, anther	O-C	4.5 2,4-D, 4.7 K	0.2 IAA, 4.7 K	–
Brassica oleracea (2)	Leaf	O-C	11.4 IAA 2.3 K	–	–
Cucurbita pepo (6)	Cotyledon, hypocotyl	SE-C	4.5 2,4-D	0.5 2,4-D	–
Sinapis alba (2)	Cotyledon, hypocotyl, stem & root	O-C	4.5 2,4-D	0.9 2,4-D 9.3 K	–
Dioscoreaceae					
Dioscorea deltoidea (2)	Tuber	O-C	4.5 2,4-D 10% CM	10% CM	0.054 NAA or –
floribunda (6)	Embryo	SE-C	4.5 2,4-D	–	0.054 NAA or –
Euphorbiaceae					
Euphorbia pulcherrima (2)	Internode, petiole	O-C	20 NAA, 2 K	10–50 2iP, 0.5 NAA	0.054 NAA

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Gramineae					
Festuca					
arundinacea (2)	Embryo	O-C	40.7 2,4-D	2.3 2,4-D	0.054 NAA or –
spp. (16)	Meristem	AS	0.93 K, 0.045 2,4-D	0.93 K 0.045 2,4-D	0.93 K 0.045 2,4-D
Hordeum					
vulgare (2)	Meristem	O-C	10 IAA, 1.5 2,4-D, 1.5 K	–	0.054 NAA or –
Oryza					
sativa (2)	Root	O-C	10 2,4-D	–	0.054 NAA or –
sativa (6)	Leaf	SE-C	4.7 2,4-D	–	0.054 NAA or –
Panicum					
maximum (2)	Inflores-cence	O-C	9.0 2,4-D	–	0.054 NAA or –
Pennisetum					
americanum (6)	Inflores-cence	SE-C	11.3 2,4-D	–	0.054 NAA or –
Saccharum spp. (2)	Leaf, inflorescence	O-C	13.6 2,4-D	0 or 26.9 NAA	0.054 NAA or –
Triticum					
aestivum (2)	Rachis, embryo, seed	O-C	4.5–9 2,4-D	4.6 Z, 5.7 IAA 1 mg/l 1 mg/l	0.054 NAA or –
Zea					
mays (2)	Mesocotyl	O-C	67.8 2,4-D	–	0.054 NAA or –
Labiatae					
Perilla					
frutescens (2)	Leaf	O-C	0.45 2,4-D	4.4 BA, 0.54 NAA	0.054 NAA
Leguminosae					
Acacia					
koa (2)	Root, sucker tip	O-C	1% CM, 11.3 2,4-D	22 BA	1 IBA
Medicago					
sativa (16)	Leaf protoplasts	SE-C	9 2,4-D, 1.2 K	0.3 NAA 2.3 BA	0.054 NAA
sativa (6)	Shoot tips	SE-C	4.5 2,4-D, 0.5 Z	0.5–4.5 2,4-D	0.054 NAA
Pisum					
sativum (2)	Epicotyl	O-C	10.7 NAA, 4.7 BA	22.2 BA, 1.1 IAA	–

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Liliaceae					
Allium sativum (2)	Leaf	O-C	0.5 2,4-D, 11.7 IAA, 9.3 K	11.4 IAA, 93 K	0.054 NAA or –
sativum (16)	Meristem	AS	–	–	0.54 NAA
Asparagus officinalis (6)	Shoot	SE-C	5.4 NAA, 4.7 K	0.5–5.7 IAA 0.44–17.7 BA	0.054 NAA
Hyacinthus orientalis (2)	Flower	O-C	0.44–1.3 BA, 0.54–1.6 NAA	13 BA, 1.6 NAA	0.054 NAA or –
Scilla sibirica (2)	Bulb, leaf, inflorescence, stem, ovary	O-C	0.69–43 NAA, 0.54–9.1 2,4-D	<11.4 IAA, <0.64 NAA	0.054 NAA –
Malvaceae					
Gossypium klotzschianum (1)	Hypocotyl	SE-C	0.5 2,4-D	11.4 IAA, 4.7 K	0.054 NAA
Passifloraceae					
Passiflora suberosa (9)	Leaf	O-C	4.4 BA, 5.4 NAA	4.4 BA, 5.4 NAA	0.054 NAA
Portulacaceae					
Mesembryanthemum floribundum (2)	Root, stem, hypocotyl	O-C	11.4 IAA 20% CM	–	0.054 NAA
Ranunculaceae					
Coptis japonica (2)	Petiole	O-C	4.5 2,4-D, 0.5K	–	0.054 NAA
Ranunculus sceleratus (6)	Mesophyll protoplasts	SE-C	5.7 NAA, 10% CM	5.7 NAA, 10% CM	0.054 NAA
Rosaceae					
Prunus amygdalis (2)	Leaf, inflorescence, embryo	O-C	26.9 NAA, 10% CW	26.9 NAA 2.3–4.7 K	0.054 NAA
Rubus spp. (2)	Shoot tip	O-D	–	0.4 BA, 0.3 GA, 4.9 IBA	0.054 NAA

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Rubiaceae					
Coffea					
arabica (1)	Leaf	SE-C	2 K, 2 2,4-D	2.5 K, 0.5 NAA	0.054 NAA
Rutaceae					
Citrus					
limettoides (2)	Stem	O-C	1.1 2,4-D	2.2 BA, 1.2 K, 1.0 IAA, 500 mg/l ME	0.054 NAA
sinensis (1)	Nucellus	SE-D	–	–	–
Scrophulariaceae					
Digitalis					
purpurea (2)	Seedling	O-C	4.5 2,4-D 0.5 K	0.6 IAA, 4.7 K	0.054 NAA
Solanaceae					
Atropa					
belladonna (6)	Leaf protoplasts	SE-C	18.5 K, 5.4 pCPA	0.5–0.9 K	–
Datura					
innoxia (2)	Stem	O-C	1 2,4-D	1 BA	–
Lycopersicon					
esculentum (2)	Leaf	O-C	0.5–34.3 IAA 1–20 BA or 9.3–18.6 K	10 BA, 0.5 NAA	10.7 NAA or 11.8 IAA
esculentum (1)	Leaf	O-D		22.8 IAA, 18.6 K	10.7 NAA or 11.8 IAA
Nicotiana					
acuminata (2)	Stem	O-C	0.45 2,4-D	10 2iP, 1 IAA	–
alata (2)	Flower branches	O-C	0.45 2,4-D	5 BA	–
rustica (2)	Shoot	O-C	0.45 2,4-D	45.7 IAA, 11.9 K	–
sylvestris (2)	Leaf	O-C	0.45 2,4-D	17.1 IAA, 0.9 K	–
tabacum (2)	Stem, leaf	O-C	0.45 2,4-D	1 IAA, 5 K	–
tabacum (1)	Leaf	O-D	–	1 IAA	–
tabacum (1)	Leaf protoplasts	SE-C	6 pCPA, 2.5 K	0.02 K	–
Solanum					
nigrum (2)	Leaf	O-C	10 IAA, 1–10 BA	10 IAA, 10 BA	–
tuberosum (2)	Shoot, stem	O-C	9.05 2,4-D	4.7–46.5 K	–
tuberosum (6)	Meristem	AS	0.05 NAA	0.05 NAA	–

Table 4. Medium Requirements for Selected Plant Species Capable of Whole Plant Regeneration *in Vitro*.

Species (Reference)	*Explant*	*Type of regeneration* [a]	*Growth regulator concentrations (μM)* [b]		
			Callus or establishment	*Shooting or embryogenesis*	*Rooting*
Petunia					
hybrida (2)	Root	O-C	0–107.4 NAA, 0.4–35.2 BA	0.3–2.7 NAA, 1.1–8.9 BA	–
hybrida (6)	Stem, leaf	SE-C	4.5 2,4-D, 0.9 BA	0.5 Z	–
Umbelliferae					
Ammi					
major (6)	Hypocotyl	SE-C	28.5 IAA	11.4 IAA	–
Anethum					
graveolens (6)	Embryo	SE-C	10.7 NAA	–	–
Carum					
carvi (6)	Petiole	SE-C	10.7 NAA	–	–
Daucus					
carota (15)	Root, stem, inflorescence, petiole, zygotic embryo	SE-C	0.45 2,4-D	–	–
Vitaceae					
Vitis					
spp. (6)	Flower, leaf	SE-C	4.5 2,4-D, 0.4 BA	10.7 NAA, 0.4 BA	0.054 NAA

[a] Types of plant regeneration are O-C, organogenesis *via* callus; O-D, organogenesis directly from explant; AS, axillary shoot production; SE-C, somatic embryogenesis *via* callus; SE-D, direct somatic embryogenesis.

[b] Murashige and Skoog basal nutrient medium employed in all cases. Abbreviations: 6-benzyl adenine, BA; casein hydrolysate, CH; coconut milk, CM; *p*-chlorophenoxyacetic acid, pCPA; 2,4-dichlorophenoxyacetic acid, 2,4-D; gibberellic acid, GA; indoleacetic acid, IAA; indolebutyric acid, IBA; isopentenyl adenine, 2iP; kinetin, K; malt extract, ME; naphthaleneacetic acid, NAA; No growth regulators added, –; tetrahydropyranyl, PBA; zeatin, Z.

from callus. Also, the ratio of auxin to cytokinin is important for the production of direct shoots from cultured explants (e.g., tobacco) (*Figure 4*). For somatic embryogenesis, transfer of callus to medium devoid of growth regulators is usually sufficient to stimulate the later stages of embryo development and subsequent germination. Meristems, shoot tips and nodal sections are cultured on medium containing low levels of auxins and cytokinins at various ratios to induce axillary bud outgrowths. Addition of other growth regulators such as abscisic acid or gibberellic acid into the culture medium is not usual but may in some cases be advantageous to promote rooting (e.g., *Citrus*) or plantlet development (e.g., carrot) (6,8).

1.4 Culture Environment

Plant tissue cultures grow differently depending on the type of culture environ-

ment they are subjected to. The intensity, type and duration of light, temperature, oxygen/carbon dioxide and other gas concentrations, and physical composition of the medium all play a role in the morphogenesis of the culture. Generally, callus proliferation occurs in the dark since light tends to promote embryogenesis, shooting and greening of the callus. Explants are frequently established under 500–1000 lux illumination intensity using a 16 h photoperiod. Light is usually supplied by cool-white or special plant growth fluorescent lamps. Later, plantlet development is enhanced by higher light intensities such as 5000–10 000 lux to promote photosynthetic leaf development and aid in the habituation required to establish the plant in the *in vivo* environment. Culture room temperature is usually maintained at around 25° C. Some species may require varying temperature treatments for optimum growth. Sub-tropical plants such as palms grow best when the temperature is set at 29° C.

2. SOMATIC EMBRYOGENESIS

Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct mode of somatic embryogenesis involves the formation of an asexual embryo from a single cell or group of cells on a part of the explant tissue without an intervening callus phase. Such occurrences are notable in *Citrus*, where pre-existing nucellar tissue cells give rise to nucellar embryos (both *in vivo* and *in vitro*), and in various cases where cultured immature embryos exhibit budding (15). Also, cases have been reported such as in *Ilex aquifolium* and *Ranunculus sceleratus*, in which the epidermal stem cells give rise to adventive embryos (15). Direct embryogenesis has been reported in some anther and protoplast cultures. However, such instances are rare in comparison with the indirect mode of embryogenesis.

The indirect mode of embryogenesis consists of establishing an explant in culture, subsequent proliferation of callus and initiation of pro-embryos [usually on medium containing a high concentration of auxin e.g., 0.45–452 μM 2,4-dichlorophenoxyacetic acid (2,4-D)] and transfer of callus to nutrient medium devoid of growth regulators in order to induce bipolar embryo formation from pro-embryo initials. When the conditions are suitable these embryos germinate to produce plantlets. Generally, only a small percentage of the cells of the explant contribute to the formation of the callus. These cells are usually located on the surface layers or are in physical contact with the nutrient medium (1). The callus is heterogeneous in nature and may be composed of numerous cell types including pro-embryo initials. Pro-embryo initials may be single cells or multicellular groups. When the callus is transferred to medium containing low auxin levels, further embryogenesis occurs to give rise to more pro-embryos, and pre-formed pro-embryo initials develop into bipolar embryos in a non-synchronised fashion.

Early investigators utilised the carrot and other members of the family Umbelliferae as source species and speculated that the phenomenon of somatic embryogenesis was localised to this family. Today, we know that it is a common phenomenon that can occur in probably all plant families. Somatic embryo formation may be achieved by proper nutrient medium employment, culture

(iii) Incubate the solution at 25°C for 5 h in the dark while agitating at 30–50 r.p.m.
(iv) Sieve the solution through a 60–80 μm stainless steel mesh and collect the filtrate containing protoplasts.
(v) Centrifuge the protoplasts for 4 min at 40–50 *g* in a sterile centrifuge tube.
(vi) Remove the supernatant with a sterile pipette. Gently resuspend the pelleted protoplasts in 4.0 ml of medium consisting of 6 mM $CaCl_2.2H_2O$, 0.7 μM $KH_2PO_4.2H_2O$, and 0.5 M glucose, pH adjusted to 5.6–5.8. Repeat the centrifugation and decantation steps.
(vii) Resuspend the washed protoplasts (~ 0.5 ml) in 2 ml of the medium used in (vi).
(viii) Place resuspended protoplasts in small droplets in a 60 x 15 mm sterile Petri dish. Use 5–10 drops per dish. Seal the dish plates with Parafilm and store in a translucent plastic box humidified by a towel moistened in 1% $CuSO_4$ solution. Incubate at 25°C under 1000 lux illumination using 16 h photoperiod conditions.
(ix) Add fresh medium to droplets every week until proliferating callus is observed.
(x) Shoot production from callus is achieved by addition of MS liquid medium containing 0.5 μM BA at 2 week intervals. Shoots should regenerate within 6 weeks.
(xi) Root the regenerated shoots by transferring to half strength MS agar medium containing 0.11 μM 3-aminopyridine.
(xii) Transfer to soil using the methods previously described.

5. PLANT REGENERATION: STATUS, PROBLEMS AND POTENTIALS

Production of adventitious roots can be achieved in a number of ways. Some species such as palms require a well developed shoot and root system prior to transplanting to soil in order to obtain a free-living plant from tissue culture. Adventitious rooting is usually easily obtained from cultured shoots or plantlets by inclusion of low levels of an auxin (e.g., 0.54 μM NAA) into the nutrient medium. Several investigators have found that reducing the MS medium composition to half strength and eliminating growth regulators enhances adventitious rooting in some species (1,2,6). Sometimes the high light intensities which stimulate photosynthetic leaf production inhibit root development. This adverse effect can be combatted by wrapping the root portion of tubes with aluminium foil or inclusion of charcoal into the nutrient medium (10). Rooting can also be induced after the shoot is introduced into non-sterile soil conditions (10,11,16). High humidity conditions or intermittent misting at 25–35°C can induce rooting of shoots for several species (16). Treating shoots with a synthetic auxin has also been found to be helpful in stimulating rooting in some species (16).

Commercial clonal propagation of plants through tissue culture techniques involves fulfilling three or more stages, as clarified by Murashige (4,7). These stages could include: Stage 1, establishment of explant in culture; Stage 2, multiplication of the propagule in culture; Stage 3, production of rooted plants

Table 5. Methods of Plant Regeneration for Agricultural and Horticultural Utilisations.

Utilisation	*Propagation method*
Clonal propagation	axillary budding from shoot tips, meristems, and nodes, organogenesis through direct shooting using a variety of explant types.
Pathogen-free plant production	axillary budding using meristems
Germplasm preservation	axillary budding using shoot tips and meristems, organogenesis through callus-derived shoots
Morphogenesis studies	axillary budding, organogenesis through callus-derived shoots and direct shooting, somatic embryogenesis
Genetic variant plant production	organogenesis through callus-derived shoots, somatic embryogenesis, and protoplast-derived plantlets

and preliminarily acclimatisation for soil conditions; Stage 4, establishment of plants into soil. An outline of the applications of each type of plant regeneration method is presented in *Table 5*. Of the various methods, axillary budding and production of shootlets directly from explants are the only reliable methods for production of clonal material, although direct organogenesis from explants is also widely used for cloning plants in large-scale applications; sometimes, however, genetically variable plants arise using this technique (4,5). Axillary shooting is also the slowest method of plant propagation. Somatic embryogenesis possesses the greatest potential for producing the highest number of plants per culture. Production of plantlets from somatic embryos has not been employed as a method of clonal propagation because of the uncertainty of their genetic nature. Similarly, plantlets produced from shoots developing from callus are not used as cloning material because of a similar problem. The production of callus with morphogenic potential is possible with almost all plant species provided an adequate explant and nutrient medium is employed. The uncertainty of the genetic nature of plants derived from callus prevents this mode of plant production from being used as a reliable method of clonal propagation. Increased aneuploidy coupled with senescence contributes to the eventual loss of morphogenic ability in callus cultures (1,2). However, this mode of plant regeneration could be employed to produce variant plant types. Other methods for producing beneficial genetic diversity in plants would be to couple tissue culture propagation with chemical mutagen and ionising radiation treatments (12). Production of plantlets from hybridised protoplasts resulting from fusion of unrelated plants or transformation of cells through the uptake of foreign DNA (Chapter 3) would be another method for inducing genetic diversity in plants.

6. CONCLUSIONS

In this chapter, the emphasis of discussion has been placed on providing several technical procedures using specific species to illustrate the methods of plant regeneration *in vitro*. It should be noted that these protocols only provide a basis of understanding with which to enter the field. Modifications to these procedures will be necessary in order to adapt them to the culture of other plants. As stated

earlier, the researcher should: (a) review the literature concerning the plant in question to determine the necessary protocol to employ (this step will save much unnecessary time conducting fruitless studies); (b) carefully select suitable explant material to initiate studies; and (c) employ adequate disinfectant methods and a suitable nutrient medium to establish cultures.

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No. 7

Callus Level and Whole Plant Microculture Selection for More Salt Tolerant Creeping Bentgrass

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Creeping bentgrass (*Agrostis palustris* Huds.) is an outstanding cool season species used for golf greens, and bowling greens maintained at 0.2 to 0.3 inches cutting height. It is one of the most saline-tolerant of the cool season turfgrasses. 'Seaside' is a creeping bentgrass cultivar widely planted near the seashore, but it can not survive on soil which contains 120 meq L^{-1} of $CaCl_2$ and NaCl (260 meq L^{-1} total salts in seawater). However, callus induction in creeping bentgrass has been achieved from mature embryos by incubating in MS media with 2,4-D as a hormone source, and regenerated plants have been obtained on hormone free MS media. However, there are no reports of successful salt tolerant whole-plant selection via cell culture of this species. In vitro selection for salt-tolerant cell lines has been conducted for tomato, wheat , barley, tobacco and Kentucky bluegrass. Single step exposure of induced callus to the selective agent has been used to select a desirable genotype. A whole plant microculture (WPMC) system has recently been developed which permits intact root growth observation through the culture medium and vessel. In this report, in order to efficiently screen for potential cell-level salt tolerance, a single-step selective method from callus culture was used to isolate putative salt tolerant lines. Plantlets were regenerated and re-screened in a WPMC system to determine if cell-level selection resulted in plants with whole plant level tolerance. The morphology of callus and plant regeneration was studied using scanning electron microscopy.

Salt screening at the callus level. Yellow callus was induced from the axes of embryos in 2,4-D treated media in 1 wk. The explants on 1 mg L^{-1} 2,4-D medium showed significantly faster growth (63.1 mm^2) than those on 5 mg L^{-1} (34.6 mm^2) and on 10 mg L^{-1} (24.8 mm^2) after 4 wk. The seeds on 2,4-D free medium only germinated and did not produce callus. At the initial stage, only watery calli were observed; compact callus was enhanced after subculture for 1 or 2 passages. This type of compact callus demonstrated a high frequency of somatic embryogenesis on plant regeneration medium. Plantlets were regenerated from both the Na_2SO_4 free (153 plants) and stress (91 plants from 0.5%, 136 plants from 1.0%, 25 plants from 1.5%) regeneration medium within 5 months. The agar solidified Na_2SO_4 stress medium supplied a simple approach to select Na_2SO_4 tolerant plants in our experiment. Our selective method could avoid the cell suspension cultural problems of lost regenerative competence in the selected cell lines.

WPMC screening test. Microculture plants could be generated on WPMC medium and the growth of roots and shoots were viewed through gelrite solidified medium. The mean root number, root area and shoot area of WPMC plants derived from seedlings, or plants regenerated from callus grown on 0, 0.5%, 1.0% or 1.5% Na_2SO_4 stress media were exhibited in Table 15. The root number of seedling plants and non-selected plants grown on Na_2SO_4 free WPMC medium were significantly greater than selected plants. However, the number of roots was not related to root area and shoot area. The root area of seedling plants was markedly lower than selected plants; similarly, the shoot area of seedling plants was markedly lower than the 1.0% Na_2SO_4 selected plants when grown on Na_2SO_4 free WPMC medium. The non-selected plants showed significantly greater root area and shoot area than 0.5% Na_2SO_4 selected plants when grown on 0.5% Na_2SO_4 stress WPMC medium, but there were not significant differences

Materials and Methods

Salt screening at the callus level: Before dehusking, seeds of Seaside creeping bentgrass were surface sterilized. Twenty dehusked caryopses were cultured on 30 ml MS medium with 30 g L^{-1} sucrose, 8 g L^{-1} agar, and 0, 1, 5, or 10 mg L^{-1} 2,4-D. There were 80 replications per treatment, and the experiment was repeated 1 time. All cultures were incubated in the dark at 24 ± 1°C for 4 wk in a growth chamber. Callus area was then determined at this stage using video image analysis. Mean separation was analyzed by LSD (Student's t) at the 5% level of significance. Since the measurement was non-intrusive, the callus was subsequently separated from the initial explant and subcultured for use in maintenance and in regeneration experiments. After 4 wk, callus was transferred to 30 ml fresh MS medium with 1 mg L^{-1} 2,4-D in 15 x 100 mm disposable petri dishes, and incubated under 24-h cool-white fluorescent lighting (36 µmol m^{-2} s^{-1}) at 26 ± 2°C. After 4 wk, green tissue was generated on the surface of the callus, 0.5 ±0.1 g of this callus was exposed to 20 ml media with 0.1 mg L^{-1} 6-(r,r-dimethylallylamine) purine (2iP) and 0, 0.5%, 1.0%, or 1.5% Na_2SO_4 in a pyrex text tube, and incubated in the same environmental conditions as for subculture. After an additional 4 wk, regenerated plantlets were transplanted into soil:peat:vermiculite (1:1:1 v:v:v) mix in 170 cm^3 plastic pots, then acclimated in a high-humidity growth chamber. After 7-d, the transplants were transferred to a glasshouse under mist (5 sec at 10 min intervals) for 1 week, then to a greenhouse bench for further growth (salt free) for 9 wks (25°C/20°C day/night). The morphological changes of callus was observed by scanning electron microscopy (SEM).

WPMC screening test: The same developmental-stage of nodal stolon segments (2.5-3.0 cm) from seedlings, non-selected plants (regenerated from callus grown on Na_2SO_4-free medium), and selected plants (regenerated from callus grown on Na_2SO_4-containing saline stress medium) was harvested from the plants in the greenhouse. Nodal explants were sterilized, and each explant was placed in a pyrex test tube containing 20 ml of media with 30 g L^{-1} sucrose, 1 mg L^{-1} indole acetic acid (IAA), 2.5 g L^{-1} gelrite, and either 0, 0.5%, 1.0%, or 1.5% of Na_2SO_4. Nodes from seedling plants were explanted only on salt free or 1.0% Na_2SO_4 medium. Nodes from selected plants from each putative salt tolerant plant were explanted on both salt free medium, and on medium with the same Na_2SO_4 concentration used during callus regeneration. Non-selected plants were explanted on all media formulations for comparison. Cultures were incubated for 4 wk under the same environmental conditions as used during plant regeneration. Evaluations of root number, root area (mm^2), and shoot area (mm^2) of each WPMC were made using video image analysis.

in root area between the two treatments. The root number of seedling plants grown on 1.0% Na_2SO_4 stress WPMC medium was significantly greater than the non-selected and 1.0% Na_2SO_4 selected plants, but the root area of seedling plants was markedly lower than for non-selected plants and 1.0% Na_2SO_4 selected plants; the shoot area of 1.0% Na_2SO_4 selected plants was significantly greater than seedling plants. The seedling plants generated only short root systems and terminated extension growth on 1.0% Na_2SO_4 stress medium after 10 to 14 days. The growth of non-selected plants and 1.5% Na_2SO_4 selected plants showed no marked differences when grown on 1.5% Na_2SO_4 stress WPMC medium. The root number of non-selected plants grown on Na_2SO_4 free WPMC medium was significantly greater than for plants grown on Na_2SO_4 stress WPMC medium. However, no marked differences in root area and shoot area were noted when plants were grown on Na_2SO_4 free and Na_2SO_4 stress WPMC medium. The Na_2SO_4 stress selected plants grown on both Na_2SO_4 free and Na_2SO_4 stress WPMC medium showed no marked differences on root number, root area, and shoot area, respectively. The putative salt tolerant regenerated plants from callus showed instability when vegetative characteristics were screened on WPMC prescreening system. The selected plants exhibited probably only salt adaptation at the callus level.

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DUPLICATE 1
AN 94:273978 SCISEARCH
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TI MERGING CALLUS LEVEL AND WHOLE-PLANT MICROCULTURE TO SELECT SALT-TOLERANT SEASIDE CREEPING BENTGRASS
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ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB More efficient methods for developing and identiyng new salt-tolerant (ST) turfgrasses are needed since salt from irrigation is accumulating in production and recreation soils. In this study, callus culture selection was combined with whole plant microculture (WPMC) screening to facilitate identifing ST 'Seaside' creeping bentgrass genotypes. Putative ST cell lines were first isolated from salt-challenged compact callus cultures grown in media containing 0-3% sodium sulfate (Na2SO4). These selected cell cultures were then regenerated and rescreened at the WPMC level in vitro to compare their vegetative growth relative to non-selected plants. Neither the selected nor the non-selected microcultured plants produced roots in an in vitro medium containing 3% Na2SO4. However, the selected plantlets exhibited slightly better root and shoot growth than non-selected plants at lower salinity (1.5-2.0% Na2SO4). This technique proved successful for screening potentially salt-tolerant turfgrasses. In addition to speeding selection, it may also prove useful in studies of whole plant ST mechanisms.

Excerpt Translation of "Heredity and Breeding of Plants", edited by Hiroshi Ikehashi, published by Yoken-do Publisher (2000)

訂正追補 植物の遺伝と育種

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る．葯培養あるいは bulbosam 法 (16.2.2) による半数体の作成とその倍化による早期固定技術は，従来の技術，たとえば世代促進と競合関係にあり，イネ・ムギでは部分的に利用されている．1978 年にインドの Bajaj らは，タバコの花粉培養に成功した．これは花粉粒を単独に培養して胚様体を誘導し，半数体を育成したものである．その後，ナタネおよびハクサイなどでも花粉培養が成功し，自殖系統の育種に利用されている．

18.2.3 プロトプラスト培養と細胞融合

植物の組織から酵素の働きにより細胞壁を除いて，裸の細胞，すなわちプロトプラスト (protoplast) を得て，これから植物体を再分化させることが多くの植物で可能となった．プロトプラストの表面はプラスに帯電しているので，互いに反撥するが，電気的処理や界面活性剤のポリエチレングリコール (PEG) により電気的反撥を抑えて融合させることができる (図 18.2)．

この応用として異種の植物の体細胞からプロトプラストを得て互いに融合させて体細胞雑種をつくることが可能となった．細胞融合による雑種は，1978 年のトマトとバレイショの雑種の育成以来注目を浴びた．これまでに，柑橘のオレンジとカラタチの雑種 (オレタチ) をはじめ，バレイショや花卉の場合など成功例はかなりある．しかし，遠縁の植物間の細胞融合による雑種組織からの再生の困難性，再生個体の不稔などまだ未解決の問題がある．

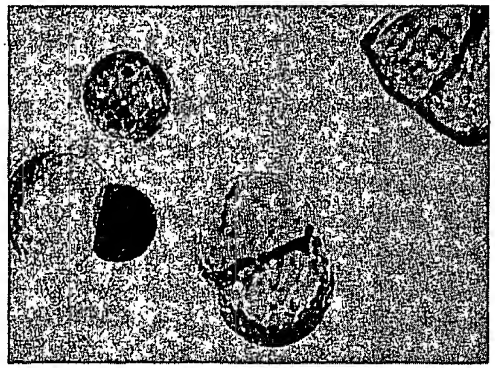

図 18.2　花弁と葉の組織からのプロトプラストの融合 (三位正洋・中野　優による)

遠縁種から望ましい一部の形質を導入するため，細胞融合の前に片方の種のプロトプラストに放射線を照射し，ゲノムの相当の部分を不活性化してから，融合すること，すなわち非対称融合も研究されている．片方の植物の核を放射線などにより不活性化してから細胞融合することにより，その細胞質のみを導入した細胞質雑種 (Cybrid) は，イネなどの細胞質雄性不稔系統の育成に利用されている．

18.2.4 細胞培養と細胞選抜

微少な未分化の細胞塊 (callus) を培地で養成して，これから植物体を再生することは比較的容易になった．その応用場面として，培地に病原菌の生産する毒素あるいは塩分を加えて，それらに対して耐性のあるカルスのみを増殖させ，それを植物体に再生させれば，与えられた毒素あるいは塩分に耐性のある植物の育成が可能と考えられた．微生物学の分野では，このような方法は早くから発達していた．たとえば突然変異の章 (7.1.2) では，Lederberg の実験を紹介した．この細胞選抜の分野は，微生物学の技術の植物育種への応用であり，1980 年前後から大きく期待された分野である．しかし多くの場合に耐性のあるカルスが得られてもそれを植物体に再分化することが困難である．また再分化した植物体では耐性が失われる場合がある．これはカルス段階のみの適応現象 (epigenetic variation の一種) である．

国際イネ研究所では，細胞選抜による耐塩性のイネ品種の育成が大規模に試みられ，いくつかの系統は得られたが，その耐塩性は既存の耐塩品種と比べて大きな差がなかった．

18.3 遺伝子の単離

有用な形質の遺伝子を植物に導入しようとするときには，その遺伝子の DNA の配列を同定しなければならない．これを遺伝子の単離 (isolation) という．微生物では，代謝突然変異の誘起と，その後の不完全培地での変異体の培養，あるいは代謝経路の分析などを通じて，遺伝形質がタンパク質のレベルで分析されてきた．したがって，遺伝形質に対応する DNA 配列の単離

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Translation of page 201, lines 7 to 21

"18.2.4 Microculture and Selection of Cells

Technique for whole plant regeneration by micro-culturing a minute undifferentiated callus in media has become relatively easier. As a practical application, it was deemed that plants, which were tolerant to pathogenic bacterial toxins or salt, would possibly be obtained by adding such toxins or salt to tissue culture media, allowing only calli tolerant thereunto to proliferate in the media, and regenerating the proliferated calli into the desired plants. In the field of microbiology, such a technique has been developed early on. For example, in the chapter 7.1.1, "Mutation", we show the experiment by Lederberg. The field of cell selection is an application of the microbiological technique to the field of plant breeding, that has been greatly expected since before or after 1980. However, in most cases, even though the desired calli tolerant to some agents, it is quite difficult to regenerate them up to the desired whole plants and also there still remains a possibility that whole plants regenerated from calli may have already lost the desired tolerance to some agents once acquired by calli. This is one of the epigenetic variations only found in the stage of callus.

International Rice Research Institute widely tried to breed salt-tolerant varieties of rice plants using the cell selection and succeeded to obtain some varieties. However, none of them showed advantageous salt-tolerance as compared to conventional ones."